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Cellular Localization and Antigenic Characterization of Crimean-Congo Hemorrhagic Fever Virus Glycoproteins

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Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus *Nairovirus* of the family *Bunyaviridae*, causes severe disease with high rates of mortality in humans. The CCHFV M RNA segment encodes the virus glycoproteins G_N and G_C. To understand the processing and intracellular localization of the CCHFV glycoproteins as well as their neutralization and protection determinants, we produced and characterized monoclonal antibodies (MAbs) specific for both G_N and G_C. Using these MAbs, we found that G_N predominantly colocalized with a Golgi marker when expressed alone or with G_C, while G_C was transported to the Golgi apparatus only in the presence of G_N. Both proteins remained endo-β-*N*-acetylglucosaminidase H sensitive, indicating that the CCHFV glycoproteins are most likely targeted to the *cis* Golgi apparatus. Golgi targeting information partly resides within the G_N ectodomain, because a soluble version of G_N lacking its transmembrane and cytoplasmic domains also localized to the Golgi apparatus. Coexpression of soluble versions of G_N and G_C also resulted in localization of soluble G_C to the Golgi apparatus, indicating that the ectodomains of these proteins are sufficient for the interactions needed for Golgi targeting. Finally, the mucin-like and P35 domains, located at the N terminus of the G_N precursor protein and removed posttranslationally by endoproteolysis, were required for Golgi targeting of G_N when it was expressed alone but were dispensable when G_C was coexpressed. In neutralization assays on SW-13 cells, MAbs to G_C, but not to G_N, prevented CCHFV infection. However, only a subset of G_C MAbs protected mice in passive-immunization experiments, while some nonneutralizing G_N MAbs efficiently protected animals from a lethal CCHFV challenge. Thus, neutralization of CCHFV likely depends not only on the properties of the antibody, but on host cell factors as well. In addition, nonneutralizing antibody-dependent mechanisms, such as antibody-dependent cell-mediated cytotoxicity, may be involved in the in vivo protection seen with the MAbs to G_C.

Crimean-Congo hemorrhagic fever virus (CCHFV) causes a hemorrhagic and toxic syndrome in humans with mortality rates of up to 50%. CCHFV infection was first described during an outbreak in Russia during the 1940s, when more than 200 cases of severe hemorrhagic fever were reported among agricultural workers and soldiers in the Crimean peninsula (15, 16). Since then, the virus has spread throughout many regions of the world, including sub-Saharan Africa (60, 61), Bulgaria, the Arabian Peninsula, Iraq, Pakistan, the former Yugoslavia, northern Greece, and northwest China (16, 23, 42–45).

CCHFV is a member of the genus *Nairovirus* within the family *Bunyaviridae* (52). Members of this enveloped-virus family have a tripartite, single-stranded RNA genome of negative polarity. The medium RNA segment (the M segment) encodes the viral glycoproteins G_N and G_C, which, like those of other *Bunyaviridae*, are synthesized as polyprotein precursors that undergo proteolytic cleavage events to yield mature glycoproteins (52). The CCHFV glycoproteins exhibit several unusual structural features and undergo several processing events. First, the CCHFV glycoproteins contain, on average, 78 to 80 cysteine residues, suggesting the presence of an exceptionally large number of disulfide bonds and a complex secondary structure. Second, the G_N precursor protein (Pre-G_N) contains a highly variable domain at its amino terminus that contains a high proportion of serine, threonine, and proline residues, and it is predicted to be heavily O glycosylated, thus resembling a mucin-like domain present in other viral glycoproteins, most notably the Ebola virus glycoprotein (56). The mucin-like region in the Ebola glycoprotein has been shown to play an important role in a cell-rounding phenotype and immunoevasion (39, 56). It is not known whether this domain plays an important role in CCHFV pathogenesis or whether it is even O glycosylated. A third unusual feature is

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that the G_N glycoprotein can undergo two posttranslational proteolytic cleavage events at the conserved motifs RSKR and RRLL, potentially releasing the mucin-like domain as well as a second N-terminal domain of approximately 35 kDa (P35, or the connector domain) (59). It is not known if the released domains traffic to an intracellular compartment, if they are secreted, or what effect they may have on viral pathogenesis and antigenic structure. Similar processing strategies have not been observed for other *Bunyaviridae* outside of the *Nairovirus* group.

As the only virally encoded membrane proteins, G_N and G_C must interact with cell surface receptors, mediate the entry of virus into cells, and serve as targets for neutralizing antibodies. Passive transfer of neutralizing antibodies can protect susceptible animals from hantavirus infection (8, 53–55, 62), and there is a report that convalescent-human sera can afford some protection in acutely infected individuals (58). Thus, characterizing the structures and functions of these proteins will be important for understanding CCHFV tropism and pathogenesis as well as for vaccine development. In this study, we describe the first monoclonal antibodies (MAbs) raised against the CCHFV glycoproteins, map the subunits to which they bind, and characterize their abilities to neutralize virus and to protect mice from a lethal CCHFV challenge. In addition, using these MAbs, we investigated the localization of G_N and G_C when expressed alone or together and have begun to map the regions involved in glycoprotein localization and interactions.

MATERIALS AND METHODS

Cells, antibodies, and viruses. CCHFV prototype strain IbAr10200, first isolated in 1976 from *Hyalomma excavatum* ticks from Sokoto, Nigeria, was grown in African green monkey kidney Vero cells or the E6 variant (51). African green monkey kidney fibroblast (CV-1), Vero, Vero E6, human cervix carcinoma (HeLa), and human embryonic kidney (HEK 293T) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Similarly, the human tumor cell line SW-13 (adrenocortical carcinoma) was grown in DMEM supplemented with 2.5% fetal bovine serum. Work with CCHFV was performed in a biosafety level 4 laboratory at the U.S. Army Medical Research Institute for Infectious Diseases. A recombinant vaccinia virus expressing the T7 bacteriophage RNA polymerase (vTF1.1) was grown in HeLa cells, and titers were determined in CV-1 cells according to standard protocols (1).

Production of MAbs. MAbs were prepared against the G_N and G_C glycoproteins of the CCHFV strain IbAr10200 by fusion of SP2/0 myeloma cells with splenocytes from BALB/c inbred mice. We carried out five independent fusions in which mice were immunized with infected suckling mouse brain homogenates (fusions I and II; MAb 30F7), with affinity-purified virion glycoproteins precipitated from nonionic detergent lysates of gradient-purified virus preparations (fusions III and IV; MAbs 1H6, 5E3, 6C2, 5B5, 8C4, 9H3, 3E3, 8G7, 8A8, and 8F10), or with affinity-purified proteins from similar lysates of infected cells (fusion V; MAbs 8A1, 12A9, 11E7, 6C11, 13G8, 14B6, 10G4, 5A5, 6B12, 10E11, 3F3, 2C9, 7F5, 11F6, and 9C6). In earlier studies, we found that hybridoma fusions carried out with splenocytes from mice immunized with mouse brain homogenates resulted predominantly in MAbs that were reactive with the viral nucleocapsid protein. To produce immunogens enriched in viral glycoproteins, cultures of SW-13 cells in 34 T-150 flasks were infected with the IbAr10200 strain of CCHFV and incubated for 36 h at 37°C. Medium fractions were removed, clarified at 10,000 × *g*, and centrifuged in an SW28 rotor (Beckman) to pellet virus particles (25,000 rpm for 3 h). Virus pellets were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 2% Triton X-100, 1% sodium deoxycholate, and protease inhibitors) and subsequently centrifuged to equilibrium in CsCl gradients. This resulted in a separation of the viral nucleocapsid (density = 1.3 g/ml) from the viral glycoproteins which banded in a broad band at lower densities. Infected-cell monolayers were similarly lysed in immunoprecipitation buffer, clarified at 10,000 × *g*, and centrifuged in an SW41 rotor (Beckman) to sediment the viral nucleocapsid from the solubilized viral glycoproteins (40,000 rpm for 4 h). Fractions enriched for viral glycoproteins from both cell and virion samples were then collected on solid-phase immunoadsorbents prepared by saturating protein A-Sepharose with anti-CCHFV hyperimmune mouse ascitic fluid (D. Watts, U.S. Army Medical Research Institute for Infectious Diseases). Immunoadsorbents were then exhaustively washed with phosphate-buffered saline (PBS), emulsified directly in complete or incomplete Freund's adjuvant, and inoculated subcutaneously and intraperitoneally into mice as described below. With this procedure, the CCHFV glycoproteins were enriched but were not pure. Importantly, the viral nucleocapsid was not detectable under these conditions, and using these immunogens, we were able to prepare a large number of CCHFV glycoprotein-specific MAbs.

MAbs were produced essentially as described previously (28). Briefly, BALB/c mice were twice immunized intraperitoneally with 100 μl of antigen preparations emulsified in Freund's complete adjuvant for primary immunization and in Freund's incomplete adjuvant for secondary immunization. Mice were euthanized 3 to 5 days after a third immunization, and splenocytes were fused with Sp2/0-Ag14 myeloma cells. Hybridoma cultures were incubated at 37°C with several changes of hypoxanthine-aminopterin-thymidine medium, and the supernatant fluids were screened by immunofluorescence (IF) assays against CCHFV-infected Vero cells and by enzyme-linked immunosorbent assays (ELISA) with viral antigen from suckling mouse brain homogenates or gradient-purified virus preparations. Antigenic specificity was initially determined by immunoprecipitation from [^{35}S]methionine-labeled infected-cell lysates (as described below) and subsequently by IF microscopy. Positive-antibody-producing cells were cloned by limiting dilution and then expanded. The immunoglobulin G subclasses of the resulting MAbs were determined by indirect ELISA analysis using hybridoma supernatants. The ELISA was developed using immunoglobulin G subclass-specific immunoglobulins (Miles Laboratories) by following the manufacturer's instructions.

Neutralization assays. Eighty percent plaque reduction neutralization (PRN-80) tests were carried out on SW-13 cell monolayers. Twofold serial dilutions of the MAbs were mixed with 200 PFU of the CCHFV IbAr10200 strain and incubated for 1 h at 37°C. Confluent monolayers of SW-13 cells in six-well plates were incubated with the virus-antibody mixture for 1 h at 37°C. The inocula were removed, and 1 ml of overlay consisting of 1 part double-strength DMEM with 5% fetal bovine serum and 1 part low-gelling-temperature agarose (Bio-Rad Laboratories, Richmond, CA) in distilled water was added. After incubation at 35°C in a sealed chamber for 2 to 5 days, the plaques were visualized by neutral red staining.

Protection studies. To evaluate the protective activities of MAbs directed against the CCHFV glycoproteins in an animal model, suckling mice, which are susceptible to infection with CCHFV (24, 50), were challenged with live virus before or after passive immunization with the CCHFV-specific MAbs. Two- to 3-day-old suckling mice were inoculated in groups of five to eight by intraperitoneal injection with 50 μl of undiluted ascitic fluid containing the different MAbs. The ascitic fluids were administrated 24 h before or after the inoculation of 100 50% lethal-dose units of the CCHFV strain IbAr10200. Ascitic fluid from Sp2/0 cells that did not contain virus-specific antibodies was used as a negative control.

Construction of CCHFV glycoprotein clones. The pCAGGS-M clone was created by cloning the entire M segment of IbAr10200 into the NruI and XhoI sites found in the plasmid pCAGGS-MCSII (41). The M segment was digested using the unique restriction enzyme sites SnaBI and SalI located in the untranslated regions of the gene. This clone was used as a template for the generation of a panel of constructs used to map functional regions on CCHFV glycoproteins (Fig. 1). Primers were synthesized according to the published sequence for IbAr10200 (51), and standard PCR technology was performed for cloning into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). The 5′-end primers included the CACC sequence at the 5′ end and the start codon to allow for directional cloning. The 3′-end primers did not possess a stop codon to allow the inclusion of the V5 and His epitope tags at the C terminus of the protein. The cloning was performed as described by the manufacturer (Invitrogen), and all constructs were sequenced. All primer sequences are available upon request.

Protein analysis. To analyze protein expression, we transfected HEK 293T cells using Lipofectamine 2000 (Invitrogen). After 24 h, cell extracts were prepared in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Cell lysates were incubated at 4°C for 3 min and then centrifuged at 10,000 × *g* for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4 to 15% Tris-HCl gels (Bio-Rad, Hercules, CA), followed by Western blot analysis with mouse anti-V5 (Invitrogen) as the primary antibody and sheep anti-mouse horseradish peroxidase conjugate as the second-

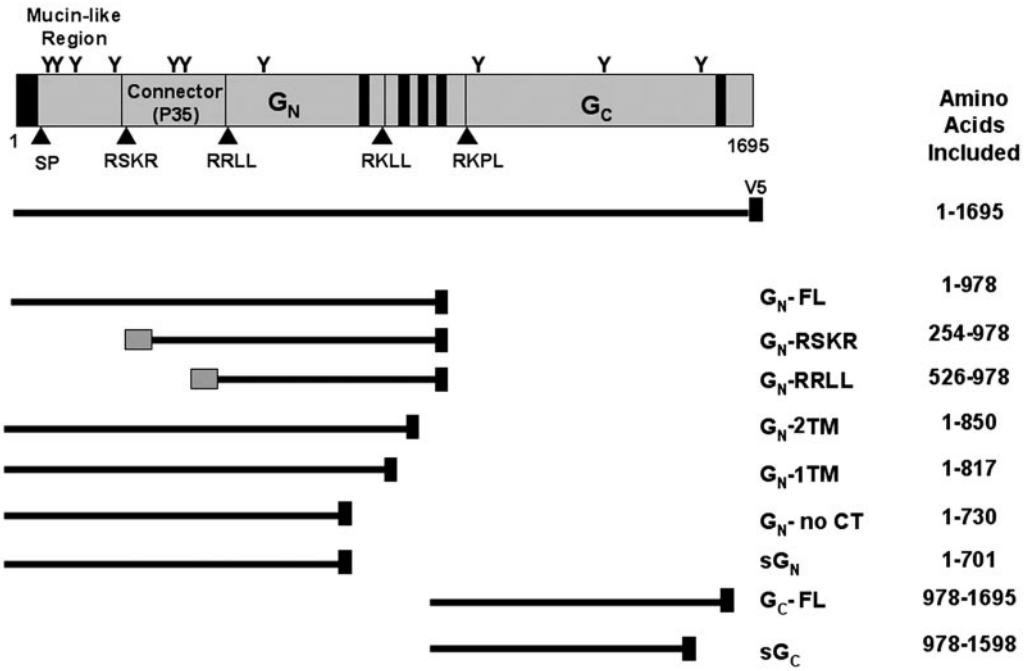

FIG. 1. Schematic representation of CCHFV M segment constructs. Predicted N-linked glycosylation sites are indicated with "Y." The filled triangles (▲) indicate the predicted conserved cleavage sites with the four conserved amino acid cleavage sites indicated (59), and the black bars indicate the predicted TMs. Specific constructs used in this study are also indicated by black lines, indicating which portions of the CCHFV M segment are included in each construct. The gray boxes on the constructs G_N-RSKR and G_N-RRLL indicate the inclusion of the hen egg white lysozyme signal peptide at the N terminus of the protein (6). A V5 epitope tag was positioned at the C terminus of each construct; these tags are represented as black boxes on the far-right side of each construct. Finally, the amino acids included in each construct are indicated on the right column, using the first methionine in the IbAr10200 strain as residue 1.

ary antibody (Amersham Pharmacia, Buckinghamshire, United Kingdom), followed by visualization with ECL-Plus Western blotting detection reagents (Bioscience, Piscataway, NJ).

IF microscopy. Localization of the CCHFV glycoproteins by indirect IF was performed as described previously (38). HeLa cells grown to 50% confluence on glass coverslips were infected with the recombinant vaccinia virus vTF1.1 (1), followed 40 min later by transfection with the different pcDNA3.1D/V5-His constructs (Fig. 1). When proteins were expressed with pCAGGS constructs, vTF1.1 infection was not utilized, since protein expression was obtained from the chicken beta-actin promoter. At 24 h posttransfection, the cells were fixed with 2% (wt/vol) paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100, and stained with a G_N- or G_C-specific MAb ascites fluid (diluted 1:250) or with mouse anti-V5 MAb (diluted 1:500) (Invitrogen) in PBS containing 0.5 mM $MgCl_2$ and 4% fetal bovine serum. In addition, TGN46, a sheep antibody specific for a heavily glycosylated protein localized primarily in the trans-Golgi network, was included as a marker for Golgi localization (Serotec, Oxford, United Kingdom). Then, cells were washed with PBS and incubated for 1 h with the secondary antibodies conjugated to Alexa Flour 488 (goat anti-mouse) and Alexa Flour 594 (goat anti-sheep) (Molecular Probes, Eugene, OR) diluted 1:500 in PBS–4% fetal bovine serum. Finally, cells were washed in PBS, mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), and examined on a Nikon E600 microscope at a magnification of ×60 utilizing UV illumination.

Endoglycosidase treatment. The method of Trimble and Maley (57) was slightly modified for digesting glycosylated CCHFV glycoproteins with endo-β-*N*-acetylglucosaminidase H (endo-H). Cell lysates from transfected HEK 293T cells were boiled for 5 min in a 50 mM sodium citrate buffer (pH 5.5) containing 1% SDS and 200 μg/ml of phenylmethylsulfonyl fluoride. After cooling, the samples were supplemented with an equal volume of 0.02 U of endo-H (New England BioLabs Inc., Beverly, MA) in a 50 mM sodium citrate buffer (pH 5.5) or the same buffer without the enzyme before incubation at 37°C for 20 h. After this incubation, the samples were analyzed by SDS-PAGE. In addition, the glycoproteins were treated with peptide-*N*-glycosidase F (PNGase F) (New England Biolabs). A similar protocol was followed for PNGase F treatment, but the digestion was performed in 100 mM sodium phosphate, pH 7.5, with 0.75% NP-40, 0.1% SDS, and 50 mM β-mercaptoethanol.

Immunoprecipitations from infected cells. Immunoprecipitation assays were performed as previously described (38). Briefly, SW-13 cell monolayers (1×10^6 cells) were infected with the CCHFV strain IbAr10200 at a multiplicity of infection of 5. Infected SW-13 cells were placed in methionine-free DMEM at 28 h postinfection for 30 min, and then 30 μCi of [^{35}S]methionine (Trans-^{35}S-label; ICN, Irvine, CA) was added. After 1 h of labeling, 50 μg of unlabeled methionine per ml was added. Infected cells were collected 24 h postlabeling or 20 h posttransfection and lysed with 0.5 ml of lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% CHAPSO {3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate}, 5 mM EDTA, and a Complete protease inhibitor mixture (Roche). Precleared lysates were incubated for 1 h at 4°C with the MAbs. Glycoproteins were immunoprecipitated using 7 μg of the MAbs generated in this study. The MAbs were previously coupled to protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitated proteins were eluted at 100°C for 5 min with 20 μl of sample buffer (0.08 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue). The immunoprecipitated proteins were detected after separation by 10% SDS-PAGE utilizing a personal densitometer (model 860; Molecular Dynamics, Inc., Sunnyvale, CA), and the data were analyzed with the ImageQuant NT software.

Immunoprecipitation from transfected cells. For the analysis of the different CCHFV glycoprotein constructs, HEK 293T cell monolayers (1×10^6) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The immunoprecipitation protocol utilized was in general the same as that described above for infected cells. The V5 epitope tag present at the C terminus of the recombinant proteins was detected using 7 μg of a MAb directed against the V5 tag (Invitrogen). Samples from transfected cells were analyzed by Western blotting as described above, using a rabbit anti-V5 tag hyperimmune serum diluted 1:5,000 (Sigma).

RESULTS

CCHFV M segment processing. Glycoproteins of the nairovirus group of bunyaviruses are processed in a distinctive and

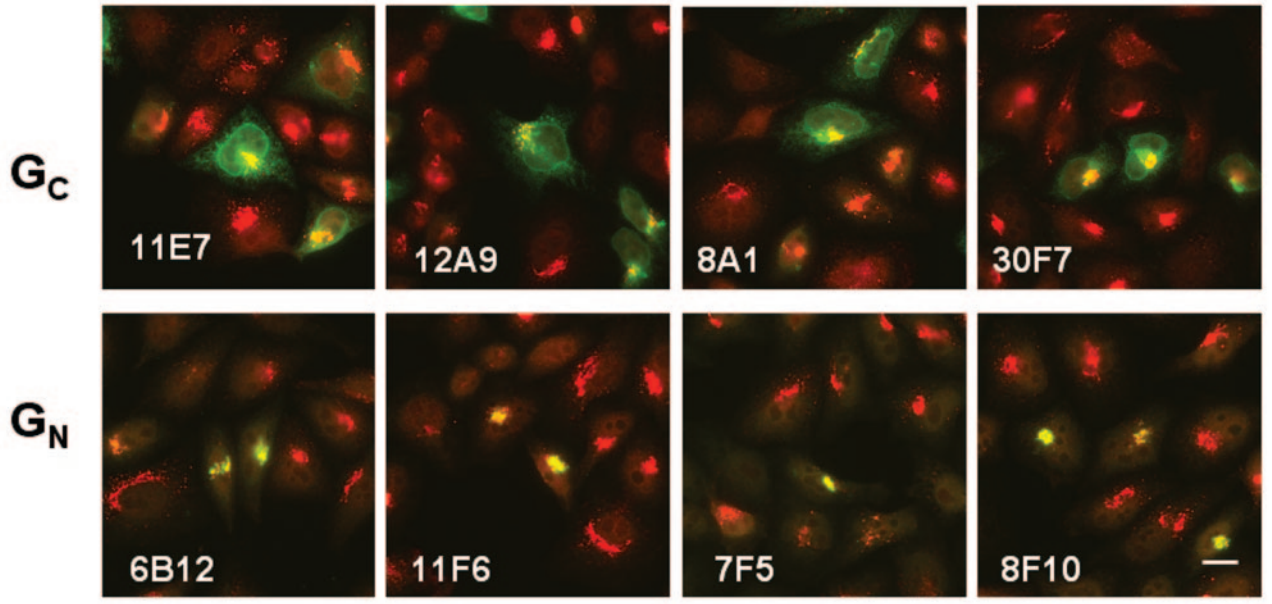

FIG. 2. Analysis of G_N and G_C cellular localization. HeLa cells were transfected with a pCAGGS vector expressing the full-length M segment of CCHFV. After 24 h, the cells were fixed and stained using the indicated MAbs in addition to an antibody specific for the Golgi resident protein TGN46 as described in Materials and Methods. The images show the Golgi apparatus (red) and the glycoproteins (green) merged. Yellow indicates colocalization. The secondary antibodies used were Alexa Fluor 488 (goat anti-mouse) and Alexa Fluor 594 (goat anti-sheep). Magnification, ×60; bar = 100 nm.

complex manner (51, 59). The M segment of CCHFV encodes a polyprotein that undergoes proteolytic processing to yield a 140-kDa Pre-G_N (termed G_N-FL in Fig. 1) and an 85-kDa precursor of G_C (Pre-G_C; termed G_C-FL in Fig. 1). Recently, it was shown that the N terminus of the mature G_N protein contains both a mucin-like and a P35 connector domain (Fig. 1) that are removed by specific cleavage with protease SKI-1, resulting in the generation of mature G_N (G_N-RRLL in Fig. 1) (59). However, how G_N and G_C are cleaved from each other is not clear, though it is thought that the N terminus of the mature G_C protein is generated by cleavage at a conserved RKPL site (59). Finally, cleavage between the mucin domain and the P35 region may be mediated by furin, which is consistent with the presence of a conserved RSKR sequence between these domains (Fig. 1) (59).

MAbs to CCHFV. To assist in our studies of CCHFV glycoprotein processing and localization, and to determine if antibodies can confer protection to CCHFV, we produced a panel of MAbs from mice immunized with a variety of CCHFV antigens as described in Materials and Methods. MAbs to CCHFV were identified by ELISA, after which both immunoprecipitation and immunofluorescence studies were done to identify the glycoprotein subunit to which each MAb bound. Only the 26 MAbs whose specificity could be clearly identified by both techniques are included in this study. For the immunofluorescence studies, HeLa cells were transfected with pcDNA3.1D/V5-His vectors expressing either G_N (G_N-FL, G_N-RSKR, or G_N-RRLL) or G_C (G_C-FL) and were infected with vTF1.1, a recombinant vaccinia virus that expresses the T7 RNA polymerase (1), in order to achieve high levels of protein expression. The cells were then fixed and processed for IF microscopy. All G_N antibodies recognized G_N-FL but most did not recognize G_N-RSKR or G_N-RRLL, with the exception of MAb 8F10, which recognized all the G_N constructs. Since neither G_N-RSKR nor G_N-RRLL was correctly transported to the Golgi apparatus (see below), the failure of most G_N MAbs to recognize these constructs could be due to protein misfolding rather than the loss of specific epitopes.

From the panel of 26 MAbs, we selected six directed against G_N and six directed against G_C for the detailed study of CCHFV glycoprotein processing and targeting. When analyzed by IF and when utilizing HeLa cells transfected with the entire M segment of CCHFV strain IbAr10200, all anti-G_C MAbs gave similar patterns, with G_C being localized to both the endoplasmic reticulum (ER) and Golgi regions as judged by colocalization with Golgi and ER markers (Fig. 2 and data not shown). The six anti-G_N MAbs recognized G_N only in the Golgi apparatus, suggesting that they recognize epitopes that are either formed after the protein reaches the Golgi apparatus or formed shortly before exit from the ER (Fig. 2). None of the MAbs detected G_N or G_C protein on the surfaces of unpermeabilized cells (data not shown). Our results indicate that the CCHFV glycoproteins are not delivered to the cell surface in appreciable quantities, which is consistent with studies that have shown that other *Bunyaviridae* bud into the Golgi apparatus and that their glycoproteins are targeted to this organelle and not delivered to the cell surface (13, 18, 29, 34, 37).

To determine if the MAbs could recognize G_N and G_C in different experimental contexts, both Western blot analysis and immunoprecipitation assay were performed. None of the MAbs recognized CCHFV proteins under fully denaturing conditions by Western blot analysis. However, in the absence of boiling, MAb 11E7 could recognize G_C obtained from transfected cells or from virus-infected cells by Western blot analysis (Fig. 3).

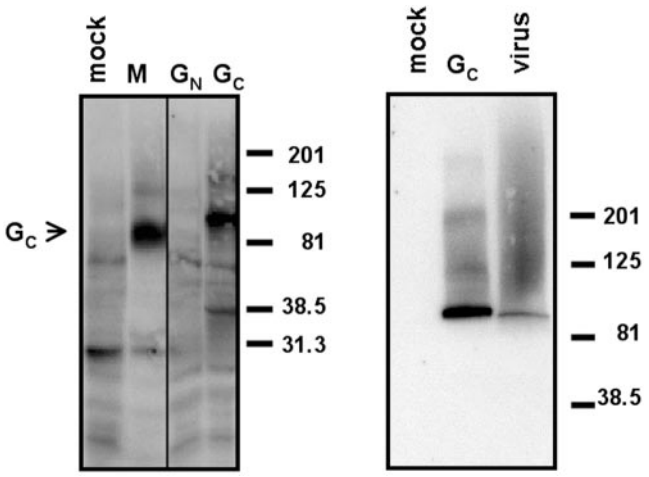

FIG. 3. MAb 11E7 directed against G_C recognizes a linear epitope. HEK 293T cells were transfected with the pCAGGS vector containing the entire CCHFV M segment (M), infected with vaccinia virus vTF1.1 and transfected with the pDNA3.1 constructs containing the G_N or G_C full-length gene with a V5 tag at the C terminus, or mock transfected. Alternatively, SW-13 cells infected with CCHFV were lysed and analyzed by Western blotting (lane labeled "virus"). Western blotting was performed utilizing MAb 11E7. Molecular size markers (in kilodaltons) are noted at the right of each blot.

The failure of most of the MAbs to recognize their antigens by Western blot analysis indicates that they bind to conformation-dependent determinants that are lost upon protein denaturation.

The MAbs were also used to immunoprecipitate G_N and G_C from lysates of cells infected with the IbAr10200 strain of CCHFV. All of the MAbs were able to immunoprecipitate the protein subunits to which they bound, which is consistent with their ability to recognize their epitopes by IF. In addition, four of the six G_C MAbs were able to immunoprecipitate both processed G_C (around 75 kDa) and its precursor protein (around 82 kDa), while all of the G_N MAbs analyzed were able to immunoprecipitate processed G_N (35 kDa) and its precursor (140 kDa), which contains both the mucin and the P35 connector domains (data not shown).

Virus neutralization and protection studies. To analyze the in vitro neutralization activities of the panel of MAbs directed against the CCHFV glycoproteins, we performed plaque reduction assays. Twofold serial dilutions of each MAb ascites fluid sample were incubated with 200 PFU of the IbAr10200 strain of CCHFV for 1 h at 37°C prior to addition to confluent SW-13 cells. Plaques were counted 3 to 5 days later, and PRN-80 titers were calculated. None of the MAbs directed against G_N exhibited neutralizing activity in this assay (Fig. 4B), though many of the MAbs directed against G_C neutralized CCHFV in vitro (Fig. 4A).

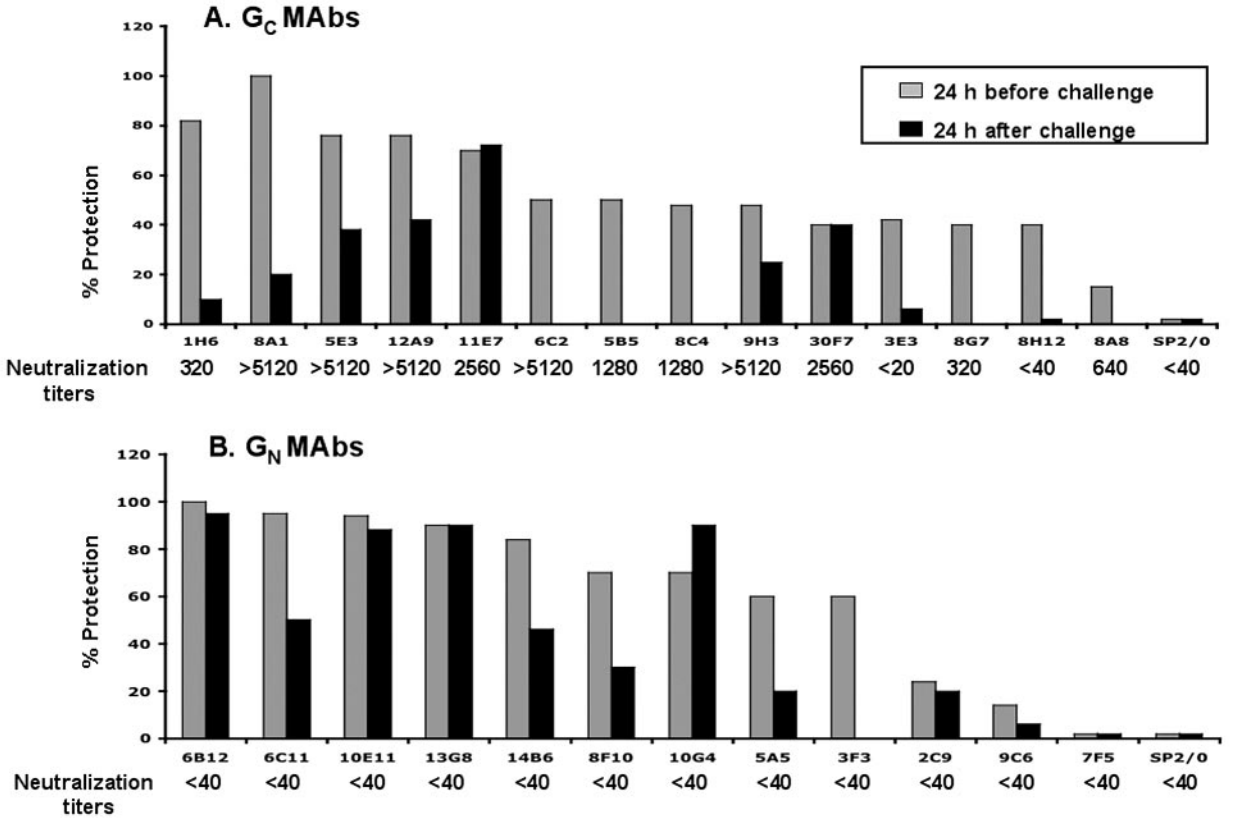

FIG. 4. Correlation between in vitro plaque reduction and in vivo protection. Passive immunization with CCHFV MAb against G_C (A) or against G_N (B). The percentage of protection represents the proportion of animals that survived challenge with respect to the total number of animals treated. Protection was determined in 2- to 3-day-old mice as described in Materials and Methods. The antibodies were provided 24 h before or 24 h after intraperitoneal challenge with the IbAr10200 strain of CCHFV. The plaque reduction neutralization titers are shown at the bottom of each plot for each antibody and represent 80% plaque reduction in SW-13 monolayer cells.

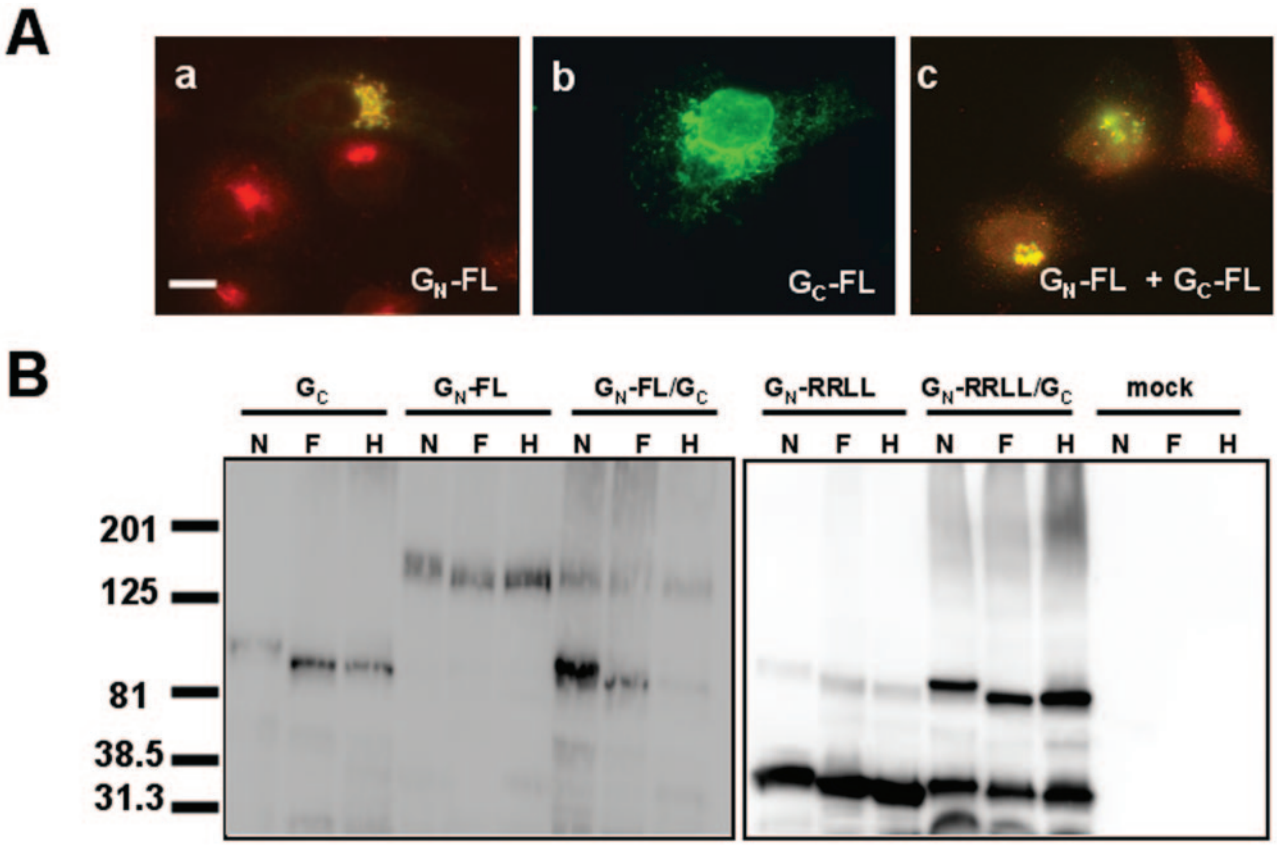

FIG. 5. (A) Localization of CCHFV glycoproteins expressed independently. HeLa cells were infected with recombinant vaccinia virus vTF1.1 and transfected with G_N-FL (a), G_C-FL (b), or both constructs (c). A schematic of the constructs is shown in Fig. 1. Twenty hours posttransfection, the cells were analyzed for the localization of the proteins by IF using MAbs specific for G_N (a) or G_C (b and c). The images show the glycoproteins (in green), the Golgi apparatus (in red), and the extent of colocalization (in yellow). Bar = 100 nm. (B) Endo-H treatment of CCHFV glycoproteins. HEK 293T cells were transfected with the G_N-FL, G_C-FL, or G_N-RRLL constructs separately or together in the presence of recombinant vaccinia virus-expressing T7 polymerase. The lysates were treated with PNGaseF (F) or endo-H (H) or were not treated (N). The lysates were analyzed by Western blotting with mouse anti-V5 antibody. Molecular size markers (in kilodaltons) are noted at the left.

Antibodies have been shown to be effective pre- and post-exposure to prophylaxis treatments for a number of viruses, including cytomegalovirus (9, 10) and respiratory syncytial virus (17, 32). Convalescent-phase serum has been shown to be of benefit to individuals acutely infected with CCHFV (57). Therefore, we tested the CCHFV MAbs for their ability to protect suckling mice challenged with the virus. The MAbs were individually administered by passive immunization to 2- to 3-day-old suckling mice either 24 h before or 24 h after challenge with 100 50% infective-dose units of the IbAr10200 strain of CCHFV (Fig. 4). Protection was registered as the percentage of animals that survived challenge with the live virus (Fig. 4). In general, anti-G_C MAbs that were capable of efficient neutralization in vitro protected mice to an appreciable degree when applied before and, to a lesser extent, after virus challenge (Fig. 4). In addition, anti-G_C MAbs that did not neutralize CCHFV infection of SW-13 cells afforded partial protection to mice from CCHFV provided that the MAbs were administered 24 h before viral challenge. When these MAbs were administered 24 h after virus challenge, protection was usually not observed. This suggests that these MAbs do possess some neutralizing activity that was not detected in our in vitro assay or that other antibody-based effector mechanisms, such as antibody-dependent cell-mediated toxicity or complement-mediated cell lysis, function in this context. Likewise, many of the anti-G_N MAbs conferred significant protection to CCHFV challenge, even when applied 24 h after virus challenge and even though they did not prevent virus infection of SW-13 cells in vitro (Fig. 4). These results show that there is an imperfect relationship between in vitro neutralization and in vivo protective ability, at least under the assay conditions used here, and that the ability of an antibody to neutralize CCHFV may depend in part on host factors, as has been observed for La Crosse virus (LACV), another bunyavirus (25–27).

G_N contains a Golgi targeting signal. To analyze the contribution of each CCHFV glycoprotein to Golgi localization, we expressed each protein independently in HeLa cells. IF analyses showed that, while G_N remained in the Golgi apparatus if expressed alone, G_C localized entirely in the ER in the absence of G_N (Fig. 5Aa and -b). When the proteins were expressed together from independent constructs, both G_C and G_N were localized in the Golgi apparatus (Fig. 5Ac). The restoration of G_C Golgi localization in the presence of G_N suggests that G_N possesses a Golgi localization signal and that G_C localizes to the Golgi apparatus through its interaction with G_N (Fig. 5A).

We further analyzed the localization of CCHFV glycoproteins when expressed independently or together by investigating their N-linked glycosylation. The G_N ectodomain of the IbAr10200 strain of CCHFV contains one predicted N-linked glycosylation site, while the G_C ectodomain contains three sites. To determine whether the CCHFV glycoproteins are glycosylated and, if so, modified by medial Golgi enzymes, we used PNGase F, which removes all N-linked carbohydrate chains, or endo-H, which removes immature carbohydrate chains. When G_C-FL was expressed alone, digestion with either endo-H or PNGase F caused the protein to migrate faster (Fig. 5B). When G_N-FL was expressed alone, it too migrated faster following digestion with either endo-H or PNGase F. To determine if the shift observed following glycosidase treatment of G_N-FL was the result of a loss of glycans on the mucin or P35 regions only, we treated the G_N-RRLL protein that lacks both the mucin and the P35 domains with endo-H. The G_N-RRLL protein also migrated faster after endo-H treatment, indicating the presence of N-linked carbohydrate chains on the ectodomain of the mature G_N as well. Similar results were obtained when the proteins were coexpressed (Fig. 5B). Thus, both proteins are N glycosylated, but neither appears to be processed in the medial Golgi apparatus when expressed alone or together.

Contribution of G_N N-terminal domains to protein localization. The CCHFV G_N protein is unusual in that it has two N-terminal domains, a mucin-like domain and the P35 domain, that appear to be cleaved from the G_N precursor in a posttranslational fashion (51). The function of these regions is unknown. A similar mucin-like domain has been found to be associated with the glycoprotein of Ebola virus and has been shown to induce cell rounding and detachment in vitro and possibly to be involved in the pathogenicity of the virus (56). To analyze the involvement of the mucin-like domain and P35 region in G_N cellular localization, we deleted these regions from constructs that contained only the G_N portion of the protein in order to generate a construct without the mucin-like domain (G_N-RSKR) and a construct without both the mucin-like domain and the P35 region (G_N-RRLL) (Fig. 1). In both cases, a signal sequence was introduced at the N terminus to ensure proper targeting to the ER. Upon expression in HeLa cells, IF analysis using a MAb to the G_N ectodomain showed that deletion of just the mucin-like domain (G_N-RSKR) resulted in a protein that localized to the Golgi apparatus in a manner similar to full-length G_N-FL (Fig. 6A). In contrast, the G_N-RRLL protein was not present in either the ER or the Golgi apparatus but was distributed in a punctuate pattern that resembled aggresomes, suggesting that it misfolds (Fig. 6B). Thus, the mucin-like domain is dispensable for Golgi targeting, while removal of the P35 connector region affected G_N localization and also led to enhanced degradation. Interestingly, localization of G_N-RRLL to the Golgi complex was recovered when the G_N-RRLL protein was coexpressed with G_C, suggesting that G_N-G_C interactions may promote the correct folding and transport of the proteins (Fig. 6C).

Mapping of the Golgi localization signal. In all *Bunyaviridae* glycoproteins that have been examined, the Golgi localization signal has been localized to G_N, generally in the cytoplasmic tail (CT) or transmembrane domain (TM) of the protein (3–5, 12, 14, 33, 34, 47, 49). The C-terminal domain of G_N contains a stretch of predicted TMs and cytoplasmic loops between the first amino acid of G_C and the predicted C terminus of G_N (59). The function of this unusual region on glycoprotein processing or any other step of the viral replication and cell cycle is unknown. To analyze the role of this region in G_N localization, we deleted two (G_N 2TM) or three (G_N 1TM) of the predicted four TMs at the G_N C terminus (Fig. 1). HeLa cells were transfected with the constructs and analyzed by IF (Fig. 6). We found that all of the C-terminally truncated G_N constructs localized to the Golgi apparatus (Fig. 6D and E). These results suggest that the Golgi localization signal is not located in this region.

Next, we designed soluble constructs that lacked the transmembrane and cytoplasmic domains of G_N (sG_N) or G_C (sG_C) or that lacked only the CT of G_N (G_N-no CT) (Fig. 1). When these constructs were expressed using the vaccinia virus T7 polymerase system in HeLa cells, both sG_N and G_N-no CT, although also present in the ER, localized to the Golgi apparatus when analyzed by IF microscopy using MAbs against G_N (Fig. 6F and G). In contrast, sG_C was restricted to the ER, just like full-length G_C (Fig. 6H). When sG_C and sG_N were coexpressed, sG_C was then targeted to the Golgi apparatus (Fig. 6I), indicating that the interaction of G_C and G_N occurs through their ectodomains, that the proteins can fold correctly when in their soluble forms, and that Golgi targeting information resides within the ectodomain of G_N.

DISCUSSION

Relatively little is known about the mechanisms by which bunyaviruses enter cells or how infection can be prevented by neutralizing antibodies, and MAbs that block CCHFV infection have not been described. Therefore, we developed a panel of MAbs to assist in our studies on CCHFV glycoprotein biology and to begin characterizing the antigenic structures of G_N and G_C. The large majority of MAbs bound to conformation-dependent epitopes in G_N or G_C. A number of MAbs against G_C, but not against G_N, were able to neutralize virus infection of SW-13 cells in vitro, suggesting that G_C plays an important role in virus entry. Similarly, MAbs directed against the G_C glycoproteins of LACV can inhibit virus infection, with some evidence indicating that this is due to a reduction in virus binding to the cell surface (19, 21, 22, 26). However, some antibodies against G_N can neutralize LACV infection in an insect cell line, though not in a mammalian cell line, suggesting that virus neutralization can be dependent on the cell type being infected and that virus entry mechanisms may differ between invertebrates and vertebrates (30). Consistent with this, proteolytic degradation of G_C with trypsin or pronase virtually eliminates the ability of LACV to bind to vertebrate, but not invertebrate, cell lines (30, 46).

Our results suggest that CCHFV neutralization mechanisms may be complex and context dependent. In general, MAbs directed against G_N were more effective at protecting mice from a lethal CCHFV challenge than were MAbs to G_C when administrated either 24 h before or after infection, even though G_N MAbs did not neutralize infection of SW-13 cells in vitro. In addition, not all G_C MAbs that neutralized CCHFV infection in vitro conferred high levels of protection in vivo, especially if administered after infection, as seen for the 8A1

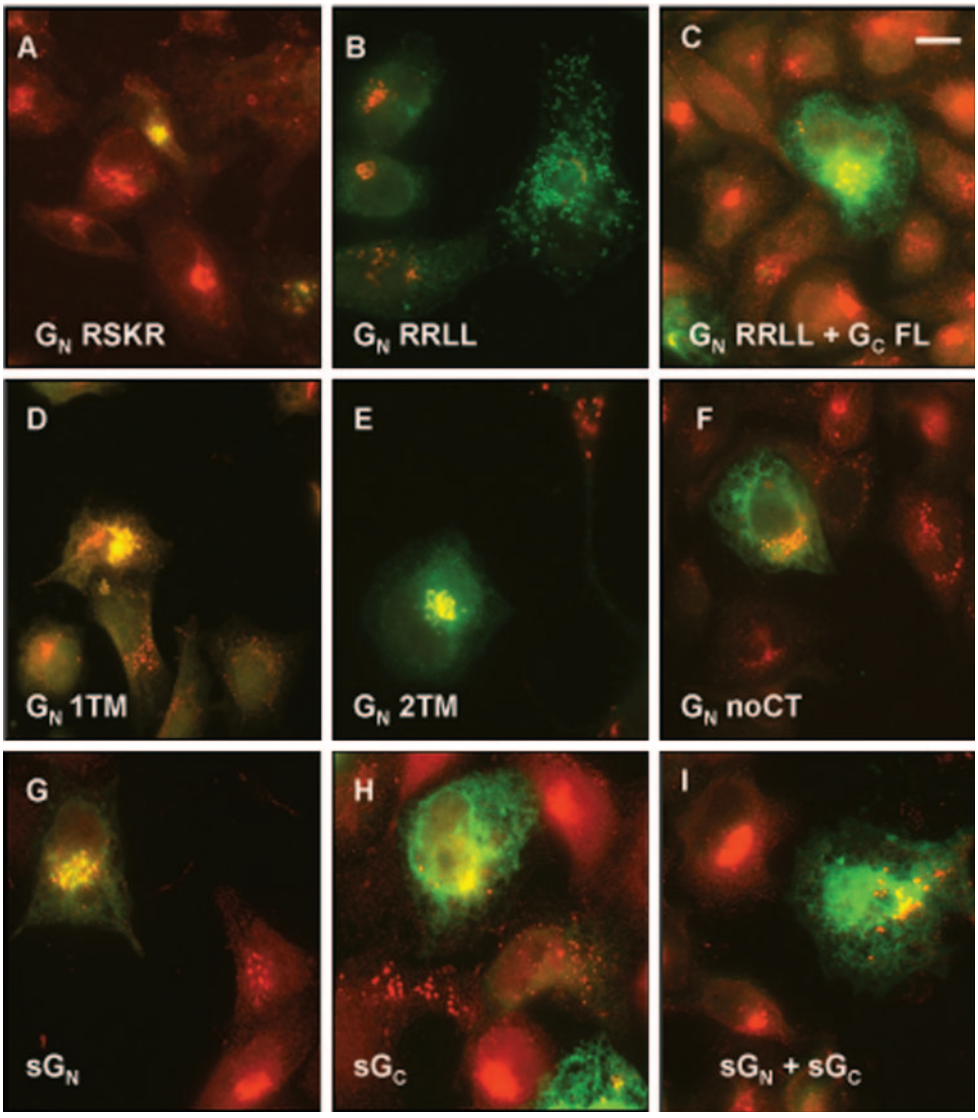

FIG. 6. Immunofluorescence analysis of the CCHFV glycoprotein constructs. HeLa cells were transfected with the indicated CCHFV constructs (shown schematically in Fig. 1) in the presence of recombinant T7 RNA polymerase expressed by vaccinia virus vTF1.1. Twenty hours posttransfection, the cells were fixed and stained using MAb 11E7 against G_C (panels H and I) and MAb 8F10 against G_N (panels A through G) (green) and the TGN46 antibody for Golgi localization (red). Nuclear staining is shown in blue. Bar = 100 nm.

and 1H6 MAbs. Thus, there was not a strict relationship between in vitro neutralization and in vivo protection. As noted above, these results resemble observations on the differential roles of LACV G_N and G_C glycoproteins in viral neutralization and protection. A soluble version of the G_C La Crosse glycoprotein is sufficient to block virus infection in mammalian cells, while antibodies against G_N neutralize infection in a mosquito cell line but not in a vertebrate cell line (31). The mechanisms that account for the differential inhibition of CCHFV infection are not currently clear. We assume that the conformational changes undergone by G_N and G_C to elicit membrane fusion and virus entry will likely be the same regardless of the cell type being infected and that these changes are likely to be induced by acid pH following endocytosis of the virus, as has been documented for La Crosse and Hantaan viruses (7, 20). However, it remains to be determined if CCHFV uses different cell surface receptors and attachment factors on different cell types. Without knowing the identity of any potential CCHFV receptors, it will be interesting to examine the ability of MAbs to neutralize CCHFV on cell lines derived from different species, including ticks.

One of the hallmarks of the *Bunyaviridae* family is that their glycoproteins are targeted to the Golgi apparatus from which they bud (48, 52). Based on this fact, a number of studies have sought to identify the signals responsible for targeting G_N and G_C to the Golgi apparatus for a number of bunyaviruses (2, 34, 36, 40, 47, 49). Generally, it has been found that the Golgi retention signals reside within the TM and/or CT of the glycoprotein closest to the N terminus of the glycoprotein precursor (18, 34, 35). However, no consensus Golgi localization motif appears to be shared among the glycoproteins of these viruses, and *Nairovirus* glycopro-

tein targeting signals and antigenic structures have not been analyzed so far.

We found that the CCHFV glycoproteins were targeted to the Golgi apparatus, as determined by IF microscopy. Since both G_N and G_C were sensitive to endo-H treatment, it is likely that the proteins are targeted to an early Golgi compartment. Similarly, hantavirus glycoproteins, although present in the Golgi apparatus, remain sensitive to endo-H treatment (49). When G_N and G_C were expressed together, G_N was localized to the Golgi complex while G_C was found in both the Golgi apparatus and the ER. It is obvious that G_N must also be present in the ER, but our conformation-dependent MAbs either do not recognize G_N in the ER or bind to epitopes that form just prior to exiting from this organelle. It is also possible that G_N is transported more quickly from the ER than G_C and, at steady state, is below our limit of detection in the ER. Indeed, some studies on the biosynthesis of Uukuniemi virus G_N and G_C proteins showed previously that G_N is transported faster than G_C from the ER to the site of virus budding at the Golgi complex (29). The apparent difference in transport kinetics is due to the fact that G_N folds and is transported from the ER to the Golgi apparatus ~30 to 45 min faster than G_C (4).

The ability of CCHFV G_N to localize to the Golgi apparatus when expressed independently of G_C indicates that G_N contains a Golgi targeting or retention motif. Since G_C is restricted to the ER in the absence of G_N, we conclude that its transport to the Golgi apparatus is dependent upon G_N and likely results from G_N-G_C oligomerization. Whether G_C fails to fold correctly in the absence of G_N, contains an ER retention signal that is masked by G_N association, or lacks a positive transport signal cannot be determined at present. Our results are in agreement with published data about other *Bunyaviridae* glycoproteins (3, 35, 36, 40, 47, 49) which localize to the Golgi apparatus in the absence of any other viral proteins (11, 14, 34, 47). Although for most of the *Bunyaviridae* glycoproteins analyzed to date, the Golgi targeting signal is contained in one of the glycoproteins, for the Hantaan virus, a member of the *Hantavirus* group, both glycoproteins are required to achieve Golgi apparatus targeting (49). Our results with CCHFV indicate that Golgi targeting information resides largely in the ectodomain of the G_N subunit, since a soluble version of G_N was largely restricted to the Golgi apparatus. However, small amounts of this protein were secreted from cells, indicating that the TM of G_N may also play a role in Golgi retention. It is also evident that the ectodomains of G_N and G_C interact with each other and that targeting of G_C to the Golgi apparatus is dependent upon its association with G_N, with ectodomain interactions being important.

In summary, although the CCHFV glycoproteins are unique in many aspects with respect to the glycoproteins from other members of the *Bunyaviridae* family, there are some similarities with regard to Golgi targeting and the glycoprotein subunits to which neutralizing antibodies are directed. Our studies indicate that CCHFV neutralization is likely to be context dependent and that more in-depth studies of various cell lines and animal models will be needed to characterize neutralization mechanisms and to identify antibodies that could be used therapeutically. Identification of regions on the CCHFV glycoproteins involved in viral neutralization, protection, and processing will contribute to our understanding of the tropism and pathogenesis of this emerging viral pathogen.

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